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Synthetic predator cues impair immune function and make the biological pesticide *Bti* more lethal for vector mosquitoes

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Abstract. The control of vector mosquitoes is one of the biggest challenges facing humankind with the use of chemical pesticides often leading to environmental impact and the evolution of resistance. Although to a lesser extent, this also holds for *Bacillus thuringiensis israelensis* (*Bti*), the most widely used biological pesticide to control mosquito populations. This raises the need for the development of integrated pest management strategies that allow the reduction of *Bti* concentrations without loss of the mosquito control efficiency. To this end, we tested in a laboratory experiment the combined effects of larval exposure to a sublethal *Bti* concentration and predation risk cues on life history and physiology of larval and adult *Culex pipiens* mosquitoes. Besides natural predator kairomones and prey alarm cues, we also tested synthetic kairomones of *Notonecta* predators. Neither *Bti* nor predation risk cues affected mortality, yet when both stressors were combined mortality increased on average by 133% compared to the treatment with only predation risk cues. This synergistic interaction was also present when *Bti* was combined with synthetic kairomones. This was further reflected in changes of the composite index of population performance, which suggested lowered per capita growth rates in mosquitoes exposed to *Bti* but only when *Bti* was combined with synthetic kairomones. Furthermore, predation risk cues shortened larval development time, reduced mass at metamorphosis in males, and had an immunosuppressive effect in larval and adult mosquitoes which may affect the mosquito vector competence. We provide the first demonstration that synthetic kairomones may generate similar effects on prey as natural kairomones. The identified immunosuppressive effect of synthetic kairomones and the novel lethal synergism type between a biological pesticide and synthetic predator kairomones provide an important proof of principle illustrating the potential of this combination for integrated mosquito control and should in a next step be evaluated under more natural conditions. It may guide novel integrated pest management programs with *Bti* that incorporate synthetic kairomones and thereby can reduce environmental impact and evolution of resistance creating more efficient and sustainable mosquito control.

Key words: Bacillus thuringiensis israelensis*; biological pesticides; ecological risk assessment; integrated pest management; mosquito control; predator stress; synergistic interactions; synthetic kairomones.*

Introduction

The control of pest species is one of the biggest challenges facing humankind (Garrett 2013, Maxmen 2013). Traditional pest control based on chemical pesticides has had success, but may be restricted by increasing concerns over environmental impact and the evolution of resistance (Heckel 2012, Köhler and Triebskorn 2013, Alphey 2014). There is indeed increasing evidence that current risk assessment strategies considerably underestimate the impact of chemical pesticides on non-target organisms (Beketov et al. 2013). Synergistic interactions of chemical pesticides with natural stressors may be an important reason for these underestimations (Coors and De Meester 2008, Holmstrup et al. 2010, Rotter et al. 2013, Vighi 2013, Dinh Van et al. 2014). Despite the potential strong effects of such synergisms on non-target organisms, the exploitation of these synergistic interactions to better control target pest species has been rarely considered (but see Birch et al. 2011).

The identification of synergisms between biological pesticides and natural stressors on pest species would be especially relevant, as biological pesticides are increasingly promoted as part of environmentally friendly integrated pest management (IPM) strategies (Lacey and Shapiro-Ilan 2008). Biological pesticides are pesticides derived from natural materials or organisms and include, for example, microbial pesticides produced from bacteria (Wilson et al. 2013). Because of their higher specificity, biological pesticides have a lower environmental impact than chemical pesticides (Lacey and Shapiro-Ilan 2008). Moreover, given the often complex mode-of-action,

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bacterial-derived biological pesticides are considered to give a lower risk for the evolution of resistance (Wirth et al. 2005, Becker et al. 2010). Yet, several studies show that also biological pesticides may have considerable impact on non-target organisms (e.g., Kanzok and Jacobs-Lorena 2006) and that target pest organisms may evolve resistance against them (Tabashnik 1994, Boyer et al. 2012). Combined exposure with natural stressors, especially when synergistic interactions occur, would allow the application of lower doses of biological pesticides, which may further reduce their environmental impact and the probability of evolving resistance (Gravitz 2012, Kroeger et al. 2013).

A widespread and potentially powerful synergism is the one between chemical pesticides and exposure to predation risk cues for which both lethal (e.g., Relyea and Mills 2001, Relyea 2003) and sublethal (e.g., Campero et al. 2007, Qin et al. 2011) effects on non-target organisms have been reported. Besides lethal, also sublethal fitness-related effects such as effects on immune function, may play an important role in population dynamics (Preisser and Bolnick 2008). Although target pest organisms may potentially also suffer from synergistic interactions between chemical pesticides and predation risk cues, this has never been studied. Furthermore, despite their potential for more efficient IPM, synergistic interactions between biological pesticides and predation risk cues have never been considered. One major limitation for the applicability of such synergisms is that predation risk cues may be difficult to manipulate at a large scale as the chemical nature is largely unknown, making it a big challenge to synthesize them. However, recently, for one important invertebrate predator, the backswimmer *Notonecta maculata*, chemical cues (i.e., kairomones) have been identified and the synthetic form has been applied successfully to repel oviposition in adult mosquitoes (Silberbush et al. 2010). This opens the possibility of combining synthetic kairomones of *Notonecta* with biological pesticides to develop a more efficient pest control program.

To address these applied ecological issues, we studied the combined lethal and sublethal effects of a biological pesticide and predation risk cues in a vector mosquito. As vector mosquito we chose *Culex pipiens molestus* (Forskal), which is very common throughout Europe and the USA where it is controlled because it causes high levels of nuisance and because it is an important vector for West Nile virus to animals and humans (Becker et al. 2010, Kilpatrick 2011). We focused on the biological pesticide *Bacillus thuringienisis* var. *israelensis* (*Bti*), a gram-positive spore-forming bacterium. *Bti* is highly specific to the larval stages of certain Diptera including mosquitoes, chironomids, and black flies that are killed by the production of protein crystals that upon ingestion lead to pore formation and cell lysis of the intestinal tract (Becker et al. 2010). *Bti* is a worldwide used biological pesticide to control vector mosquitoes in the larval stage and is the only allowed biological pesticide to fight mosquitoes in Europe (EU Biocidal Products Directive 98/8/EC). Microbial-control agents like *Bti* have been successfully used for about 30 yr in nuisance insect control programs and since the beginning of the new millennium also to control mosquitoes transmitting West Nile virus. For instance, in Quebec, Canada, more than 50 tons of *Bti* formulations are used every year (Becker et al. 2010). Given its high specificity, *Bti* has less environmental impact than chemical pesticides, yet negative effects on non-dipterans have been documented (e.g., Boisvert and Boisvert 2000, Poulin et al. 2010, Lajmanovich et al. 2015, but see Lagadic et al. 2014) and resistance may evolve (e.g., Boyer et al. 2012). Moreover, *Bti* is less efficient than chemical pesticides and is therefore sometimes used in combination with those to increase its efficacy in controlling mosquitoes (Tetreau et al. 2013). As *Notonecta* are important natural predators that may control mosquito populations (Becker et al. 2010) and that evoke antipredator responses in larval *C. pipiens* (Sih 1986), we tested for synergistic interactions between the recently discovered synthetic kairomones of *N. maculata* and *Bti* and compared the impact of natural vs. synthetic kairomones on larval prey.

We focused not only on mortality but also on sublethal life history effects (development time and mass at emergence), as these can also strongly affect vector competence, the ability of a vector to become infectious, and subsequently transmit a pathogen (Alto and Lounibos 2013). To get an integrated estimate that combines effects on these different life history traits we calculated the composite index of population performance (r'; Livdahl and Sugihara 1984, Juliano 1998). While it is well-documented that *Bti* is lethal for *Culex* larvae (e.g., Boisvert and Boisvert 2000), its effects on sublethal response variables are unknown for any dipteran species. Given that synergistic effects may only be visible for fitness-related physiological variables (Janssens and Stoks 2013a) and that sublethal fitness-related effects across metamorphosis may play an important role in population dynamics (Preisser and Bolnick 2008), we also quantified effects on fat content and immune function both in the larval and in the adult stage. As a measure of immune function, we measured the activity of phenoloxidase (PO), a key component of the insect immune system (Sugumaran 2002). The PO cascade pathway is involved in fighting against a wide range of parasites in mosquitoes (Christensen et al. 2005), indicating that stressors affecting this pathway, may also have effects on vector competence (Cornet et al. 2013).

Methods

Bti application

We used the commercial *Bti* formulation Vectobac (WG, 37.4% *Bti* at 3000 ITU/mg; Valent Biosciences, Libertyville, Illinois, USA). The *Bti* concentration for the exposure experiment was based on a range finding experiment in the laboratory where fourth instar (L4) larvae were exposed to a range of *Bti* concentrations (0, 2, 6, 10, 14, 18 μg/L) for 4 d, administered in two pulses on the first and third day. The concentrations for this range finding were based on the LC_{50} value for fourth instar *C. pipiens*

larvae (35.33 μg/L; Boudjelida et al. 2008). No mortality was observed at 10 μg/L and 30% mortality at 14 μg/L. Therefore, we selected the intermediate concentration of 12 μg/L to screen for effects on life history and carry-over effects to the adult stage. High lethal concentrations of a pesticide may reduce the possibility of detecting synergistic interactions with predator cues (Relyea and Mills 2001). Recommended dosage for Vectobac WG for mosquito control range from 1 to 5 mg/L for container habitats and from 12.5 to 75 mg/m^2 for open habitats (*data available online*).[2] If we assume a depth of 0.5 m for the typical shallow ponds that *Culex* prefers (Becker et al. 2010), this corresponds to a recommended application concentration between 25 and 150 μg/L. A stock solution of 1 mg/mL *Bti* was prepared in milliQ water.

Experimental design

To test for the effect of *Bti* exposure and predation risk cues on life history and physiology, we set up a laboratory experiment where larvae were exposed during 4 d to one of the eight combinations of two *Bti* concentrations (0 and 12 μg/L) and four predation risk cues treatments (no kairomones,No; synthetic kairomones, SK; natural kairomones, NK; and natural kairomones plus alarm cues from conspecifics, NK + AC). The latter was added because it reflects the highest dose of risk for mosquitoes (Ferrari et al. 2008) and therefore allows comparing the effect of synthetic kairomones with the strongest risk cocktail present in nature. Note that we did not include a treatment only containing alarm cues as it was not our aim to test the pure effect of alarm cues, as these normally do not occur in isolation in nature and cannot be produced synthetically. The number of replicated jars per combination of *Bti* concentration × predation risk cues treatment was 10 with each jar containing 20 larvae (total of 1600 larvae in 80 jars). Exact sample sizes per response variable are given in Appendix S4.

During the pre-exposure period, larvae were reared under the same conditions as the larvae from the culture (see Appendix S1) and daily fed 5 mL of a 25 mg/mL solution of the same food mixture used for the laboratory culture. This amount equals 0.313 mg of food/d.larva and equals a high food condition (Beketov and Liess 2007) thereby avoiding effects of food shortage on the measured response variables.

When larvae molted into the L4, we started the 4-d exposure period to *Bti* and predation risk cues. We chose a 4-d exposure period as at day five the first larvae started to pupate. At the start, 20 L4 larvae (<24 h in L4) were transferred to a 210-mL glass jar. Each jar was filled with 100 mL of one of the two *Bti* solutions (0 or 12 μg/L in dechlorinated tap water) and 1×10^7 cells of *Scenedesmus obliquus* algae. The medium was refreshed after 48 h. At the end of the 4-d exposure period, larvae were transferred to a new glass jar with 100 mL of dechlorinated tap water where they maintained until they reached metamorphosis. During the 4-d exposure period and the remaining time until metamorphosis, larvae were fed daily with the same amount of food as during the pre-exposure period.

To install the predation risk cues treatment during the 4-d exposure period, we added medium daily according to the jar treatment. To prepare the medium with natural kairomones of *N. maculata*, two bugs were starved for 96 h in their holding aquarium (see Appendix S1), after which we transferred each bug to a 50-mL glass jar with 35 mL of dechlorinated tap water for an additional starvation period of 24 h. This way no chemical cues of the prey were present in the medium. From this medium, we used 20 mL, 10 mL/predator. We pipetted 670 μL of this medium into jars from the corresponding predation risk cues treatment. This matches the realistic kairomone concentrations associated with three individual *N. maculata*/15 L in natural ponds (Silberbush et al. 2010).

To obtain medium with natural kairomones and alarm cues from conspecifics, we offered two L4 larvae from the culture to the same two bugs previously used to obtain natural kairomones. After the predator finished preying upon the larvae, 40 mL of this medium was taken (20 mL/bug). Additionally, four extra *C. pipiens* larvae were homogenized in this medium to make the alarm cues stronger (Pestana et al. 2009). From this final mixture, we added 670 μL to each jar. The same amount of dechlorinated tap water was added to the predation risk cues treatments without predator kairomones (control) and with synthetic kairomones.

As synthetic predator kairomones, we used a mixture of tricosane and heneicosane, the two hydrocarbons that were identified as kairomones of *N. maculata* (Silberbush et al. 2010). For both hydrocarbons, we obtained a stock solution of 1 mg/mL by dissolving granules obtained from Sigma-Aldrich (St. Louis, Missouri, USA) in 1,4-dioxaan (100%). From these primary stock solutions a secondary stock in milliQ water was prepared to obtain a 1 μg/mL tricosane solution and a 0.1 μg/mL heneicosane solution. We daily added 20 μL of the secondary tricosane stock solution and 33 μL of the secondary heneicosane stock solution to the corresponding jars giving concentrations of 33 ng/L and 200 ng/L, respectively. The used concentrations of tricosane and heneicosane in this treatment thereby matched the amount of natural kairomones used in the other predation risk cues treatments (based on Silberbush et al. 2010). The same amount of 1,4-dioxaan was added to all jars of the other predation risk cues treatments.

Response variables

Mortality was expressed as the total number of larvae that died/jar during the fourth larval instar. At the end of the 4-d exposure period, four larvae per jar were randomly selected, weighed to the nearest 0.01 mg using an electronic balance (AB135 S, Mettler Toledo, Zaventem, Belgium), and frozen at −80°C for later physiological analyses. Development times were calculated as the average time from the start of the L4 stage until metamorphosis of all

[2] http://www.who.int/whopes/Mosquito_Larvicides_sep_2011.pdf

surviving larvae per jar. After metamorphosis, adult mosquitoes were collected, weighed to the nearest 0.01 mg, and frozen at −80°C for physiological analyses.

To investigate how the combined effects on all measured life history traits would affect population growth rate, we calculated the composite index of population performance (r') based on Livdahl and Sugihara (1984). This index estimates the realized per capita rate of population change. We calculated r' for each jar as follows:

$$r' = \frac{\ln\left[\left(\frac{1}{N_0}\right)\sum_x A_x f(w_x)\right]}{D + \left[\sum_x x A_s f(w_x) / \sum_x A_x f(w_x)\right]}$$

N_0 is the initial number of females in each jar. We calculated N_0 separately for each replicate jar as the total number of females that emerged plus half of the animals that died prior to eclosion in that replicate. Because for these animals no sex was determined, we assumed a sex ratio of 50%. A_x is the number of females eclosing on day x, w_x is the mean size of females eclosing on day x, $f(w_x)$ is a function relating fecundity to female size, and D is the time (in days) between adult eclosion and reproduction. For *C. pipiens molestus*, D is approximately 8 d (Vinogradova 2000) and the size–fecundity relationship is $f(w_x) = 32.88\, w_x - 89.72$ ($r^2 = 0.99$) with w_x = wing length in mm (Costanzo et al. 2011, calculated from Vinogradova 2000). The analysis of female wing length is included in Appendix S2.

To quantify physiological variables, we homogenized per jar following sets of animals: two sets of two L4 larvae (both sets were averaged per jar for statistical analyses), a set of two adult females, and a set of three adult males (more males were pooled given their lower mass). Animals were homogenized using a pestle, diluted 15 times in phosphate buffer saline (PBS; pH 7.4, 100 mmol PBS), and centrifuged for 5 min (13 200 rpm; 4°C).

As a measure of immune function we quantified the activity of a key enzyme in insect immunity, phenoloxidase (PO; Sugumaran 2002, Christensen et al. 2005). PO produces indole groups, which are subsequently polymerized to melanin. The enzymatic reactions in turn produce a set of intermediate products such as quinones, diphenols, superoxide, hydrogen peroxide, and reactive nitrogen intermediates, which are important during defense against bacterial, fungal, and viral agents (González-Santoyo and Córdoba-Aguillar 2011). PO was quantified using a spectrophotometric assay based on the protocol by Stoks et al. (2006), for details see Appendix S3. PO activity was expressed per minute and per μg protein. Total fat was measured based on the protocol of Bligh and Dyer (1959), for details see Appendix S3. Fat content was expressed as μg fat/mg wet mass.

Statistical analyses

All statistical analyses were performed using jars as replicates, hence on jar totals (mortality) or jar means (other traits). Mortality was analyzed using a generalized linear model with a Poisson error distribution and the log link function with *Bti* exposure and the predation risk cues treatment as independent variables. We tested for effects of *Bti* exposure and predation risk cues on r', larval PO activity, and larval fat content using separate two-way ANOVAs.

For the variables larval development time, adult mass, adult PO activity, and adult total fat, we had information on the sex of the animals, hence we calculated separate jar means for males and females. We tested for effects of *Bti* exposure and predation risk cues on these variables by means of separated repeated-measures ANOVAs, with *Bti* exposure and predation risk cues as independent factors. Sets of means of males and females per jar were considered as repeats of that jar. This way the coupling of observations for males and females of the same jar was taken into account. For the physiological variables PO activity and fat content sample sizes are lower for some treatment combinations because in the repeated-measures ANOVA only jar replicates with data for both males and females are used (exact sample sizes are given in Appendix S4 for all variables where the number of jar replicates deviates from 10). These were not always available since two females and three males were needed in the pooled samples for physiological analysis. All observed effects for these two variables, however, remained present when we ran separate ANOVAs for males and females, including all data.

All analyses were performed in STATISTICA v12 (StatSoft, Tulsa, Oklahoma, USA). When effects were identified for the predation risk cues treatment or its interactions with *Bti*, we carried out contrast analyses to identify which treatments differed from the control treatment. For mortality, we also ran separate analyses per predation risk cues treatment to quantify the effect of *Bti* exposure since contrast analyses are not available for generalized linear models. We define a synergism based on the additive effects model when the combined effect is greater than the sum of effects elicited by the individual stressors (Folt et al. 1999). This can be tested formally as a significant interaction term in the ANOVAs. Results of contrast analyses are indicated in the corresponding figures.

Results

Life history responses

While exposure to *Bti* increased mortality, this strongly depended upon the presence of predation risk cues (Table 1, Fig. 1). Separate analyses per predation risk cues treatment showed that mortality increased on average by 133% (mean across all three treatments with predation risk cues) when both stressors were combined compared to the associated predation risk cues treatments in the absence of *Bti* (*Bti* effect for each of the three treatments with kairomones, all $P < 0.001$). This increase in mortality was not observed in the absence of predation risk cues (Wald statistic = 0.55, df = 1, $P = 0.46$). This was further indicated by the nearly significant *Bti* × predation risk cues interaction ($P = 0.059$; Table 1).

TABLE 1. Results of the general linear models testing for the effects of *Bti* and the predation risk cues on mortality and development time during the fourth larval instar, adult body mass at metamorphosis and the composite index of population performance (r') of *Culex pipiens*.

Effect	Mortality			Development time			Adult mass			r'		
	df	Wald stat.	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Bti	1	32.17	<0.001	1,64	0.03	0.853	1,61	5.10	0.027	1,66	8.57	0.005
Predation risk cues	3	4.33	0.228	3,64	4.82	0.004	3,61	1.88	0.143	3,66	0.32	0.811
Bti × predation risk cues	3	7.44	0.059	3,64	0.32	0.812	3,61	4.68	0.005	3,66	2.66	0.056
Sex				1,64	277.68	<0.001	1,61	1008.51	<0.001			
Sex × *Bti*				1,64	4.50	0.038	1,61	0.12	0.728			
Sex × predation risk cues				3,64	1.00	0.400	3,61	2.52	0.066			
Sex × *Bti* × predation risk cues				3,64	1.72	0.172	3,61	0.81	0.492			

Notes: For the analyses on development time and adult mass, sex was also included in the model.

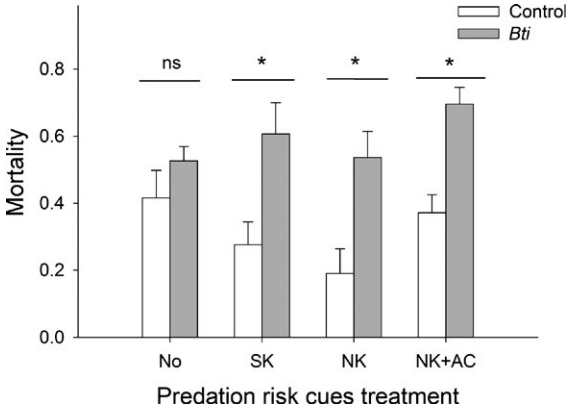

FIG. 1. Mortality of *Culex pipiens* larvae during the fourth larval instar in response to *Bti* and four predation risk cues treatments: no chemical cues (No), synthetic kairomones of *Notonecta maculata* (SK), natural kairomones of *N. maculata* (NK), and natural kairomones of *N. maculata* + *C. pipiens* alarm cues (NK + AC). Mortality is expressed as the log-transformed (x + 1) number of larvae that died. Jar means are given with 1 SE (based on 10 jar replicates). Lines represent contrast analyses testing for the effect of the *Bti* treatment per predation risk cues treatment (* $P < 0.05$, ns $P > 0.05$).

The predation risk cues treatment had an overall effect on development time (Table 1; Fig. 2A, B). Larvae exposed to the combination of natural kairomones and alarm cues had a significantly shorter development time than the other three predation risk cues treatments, including the control with no predator kairomones (contrast analyses, all $P < 0.034$; Fig. 2A, B). Males had ~3 d (18%) shorter development times than females (Table 1, Fig. 2A, B). Although exposure to *Bti* had no overall effect on the development time, both sexes did react in a different way to *Bti*, with females, if anything, slightly tending to accelerate development and males slightly tending to decelerate development (sex × *Bti*; Table 1, Fig. 2A, B).

Exposure to *Bti* increased mass at metamorphosis, but only in adults reared as larvae in the presence of natural kairomones (*Bti* × predation risk cues; Table 1, Fig. 2C, D; contrast analyses, *Bti* effect with natural kairomones, $F_{1,61} = 18.35$, $P < 0.001$; *Bti* effect for the other predation risk cues treatments, all $P > 0.90$). Females were heavier than males (Fig. 2C, D). Males exposed as larvae to the cocktail of natural kairomones and alarm cues had a lower mass compared to the control treatment without predator kairomones (contrast analysis, $F_{1,61} = 11$, $P = 0.0015$), while females did not show this mass reduction ($F_{1,61} = 0.0063$, $P > 0.94$; sex × predation risk cues; Table 1).

While exposure to *Bti* resulted in a lower composite index of population performance (r'), this strongly depended upon the presence of predation risk cues (Table 1, Fig. 3). Separate analyses per predation risk cues treatment showed that this *Bti*-induced reduction of r' was only present for the treatment with synthetic predator kairomones ($P = 0.003$) and for the combination of natural kairomones plus alarm cues ($P = 0.014$). This reduction was not observed in the absence of predation risk cues ($P = 0.77$) and also not in the presence of only natural kairomones ($P = 0.63$). This was further indicated by the nearly significant *Bti* × predation risk cues interaction ($P = 0.056$; Table 1).

Physiological responses

Larvae exposed to the cocktail of natural kairomones and alarm cues had a lower PO activity compared to control larvae (contrast analysis, $F_{1,72} = 5.52$, $P = 0.022$; Fig. 4A; effect of the other predation risk cues treatments, all $P > 0.058$; main effect predation risk cues, $P = 0.07$; Table 2). Exposure to neither *Bti* nor predation risk cues had an effect on the fat content of larvae (Table 2, Fig. 4B).

The predation risk cues treatment had an overall negative effect on the PO activity in adult mosquitoes (Table 2, Fig. 5A, B). Adults exposed to predation risk cues as larvae had a lower PO activity compared to adult mosquitoes from the control treatment without predation risk cues

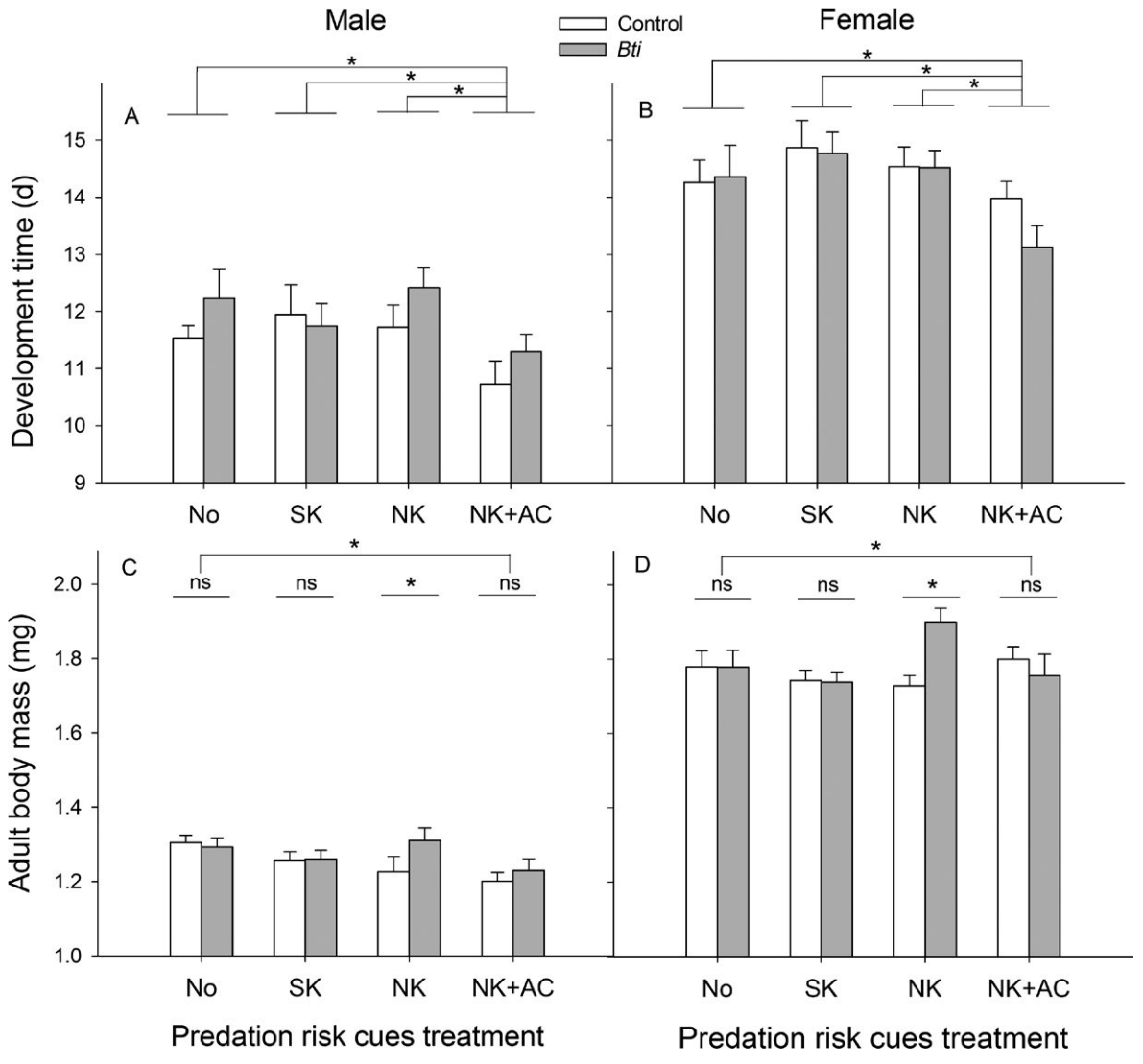

FIG. 2. (A, B) Development time and (C, D) adult body mass of *Culex pipiens* mosquitoes in response to *Bti* and four predation risk cues treatments: no chemical cues (No), synthetic kairomones of *Notonecta maculata* (SK), natural kairomones of *N. maculata* (NK), and natural kairomones of *N. maculata* + *C. pipiens* alarm cues (NK + AC). Given are jar means with 1 SE for (A, C) males and (B, D) females. Lines represent contrast analyses explained in the results section.

(contrast analyses, all $P < 0.037$; Fig. 5A, B). Exposure to neither *Bti* nor predation risk cues had an effect on the fat content of adult mosquitoes (Table 2; Fig. 5C, D). Females had a lower fat content than males (Table 2; Fig. 5C, D).

DISCUSSION

Responses to isolated exposure to predation risk cues

Our results indicate that predation risk cues had effects on life history (development time and mass at metamorphosis) as well as on physiology (PO activity) in larval and adult mosquitoes. These effects were often only present when predator kairomones were combined with alarm cues, reflecting the highest dose of risk for prey organisms, thereby confirming that prey may respond in a threat-sensitive way to kairomones associated with predation risk (Pestana et al. 2009, for *Culex* mosquitoes, Sih 1986, Ferrari et al. 2008).

In the presence of the cocktail of natural kairomones and prey alarm cues, the *Culex* larvae accelerated development, possibly to escape the risk of being preyed upon by aquatic predators (Higginson and Ruxton 2010). While a longer predator-induced development time has been observed for mosquito species (Beketov and Liess 2007, van Uitregt et al. 2012), studies on *Culex* mosquitoes (Silberbush et al., *unpublished manuscript*), and other aquatic insects also found an accelerated life history when prey were exposed to predation risk cues (e.g., Dahl and

TABLE 2. Results of the general linear models testing for the effects of *Bti* and predation risk cues on phenoloxidase (PO) activity and total fat content in larvae and adults of *Culex pipiens*.

Effect	PO activity larvae			Total fat larvae			PO activity adults			Total fat adults		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Bti	1,72	1.21	0.275	1,72	0.40	0.532	1,46	0.80	0.375	1,44	0.52	0.476
Predation risk cues	3,72	2.46	0.070	3,72	1.20	0.318	3,46	4.16	0.011	3,44	0.99	0.408
Bti × predation risk cues	3,72	0.16	0.924	3,72	0.31	0.822	3,46	1.37	0.264	3,44	1.45	0.242
Sex							1,46	0.06	0.814	1,44	9.89	0.003
Sex × *Bti*							1,46	0.39	0.536	1,44	0.82	0.371
Sex × predation risk cues							3,46	1.69	0.184	3,44	1.04	0.385
Sex × *Bti* × predation risk cues							3,46	0.44	0.729	3,44	1.40	0.255

Notes: For the analyses on adult PO activity and total fat also sex was included in the model.

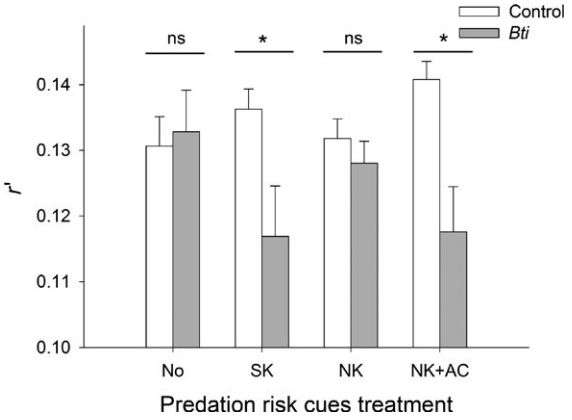

FIG. 3. Mean (with 1 SE) composite index of population performance (r') of *Culex pipiens* in response to *Bti* and four predation risk cues treatments: no chemical cues (No), synthetic kairomones of *Notonecta maculata* (SK), natural kairomones of *N. maculata* (NK), and natural kairomones of *N. maculata* + *C. pipiens* alarm cues (NK + AC). Lines represent contrast analyses testing for the effect of the *Bti* treatment per predation risk cues treatment (* $P < 0.05$, ns $P > 0.05$)

Peckarsky 2003, Stoks et al. 2012). Possibly this faster development was reached by a re-allocation of energy away from other functions like immune function (Stoks et al. 2012). In line with this, PO activity was reduced in larvae and adults exposed to the cocktail of natural predator kairomones and alarm cues; such predator-induced immunosuppression has been observed before in other taxa (e.g., Stoks et al. 2006, Groner et al. 2014). In adults, this reduction in PO activity was also present when they had been exposed as larvae only to natural or synthetic kairomones (predation risk cues that did not evoke a faster development), suggesting that besides food re-allocation toward an accelerated development, also a reduced food acquisition may have contributed to the immunosuppression. Also the observed pattern in mass at emergence, being lower when adult males were exposed to the combination of natural kairomones and alarm cues, supports previous work in aquatic organisms (e.g., Dahl and Peckarsky 2003), including mosquitoes (e.g., van Uitregt et al. 2012). The reduced mass of adult males can be explained by the observed shorter development time without a compensatory reaction in growth rate (results not shown). Possibly the mass reduction was only present in males as these had a considerably shorter development time than females.

The observed predator-induced reductions of immune function and adult mass (in males) likely have a negative influence on the adult fitness, such as a lower resistance to starvation (van Uitregt et al. 2012), a shorter adult lifespan, and a lower flight capacity (Briegel et al. 2001). Carry-over effects across metamorphosis of larval stressors, such as predation risk on adult fitness-related traits, are increasingly reported in other taxa (e.g., Stoks et al. 2006, Groner et al. 2014) and may be an important factor negatively affecting prey population dynamics (Preisser and Bolnick 2008).

The identified immuno-suppressive and life history effects can also influence vector competence and can therefore be important in disease transmission. Vector competence has two components (Breaux et al. 2014): physiological vector competence, dealing with physiological traits involved in the host-pathogen interaction (e.g., immune and defense mechanisms), and functional vector competence, dealing with traits contributing to the ability of an individual mosquito to transmit the pathogen effectively given that it has attained physiological competence (e.g., flight capacity, longevity, and host localization). The net effect of larval stressors on adult vector competence will critically depend on the strength and direction both components will be affected and the resulting balance is hard to predict (Breaux et al. 2014). On the one hand, the here-observed reduced immune function under predation risk can result in more susceptible adults, with a greater proportion of stressed mosquitoes becoming competent vectors (Alto et al. 2008, Juliano 2009). On the other hand, the reduced immune function and body mass can reduce the longevity and flight capacity, hence the functional

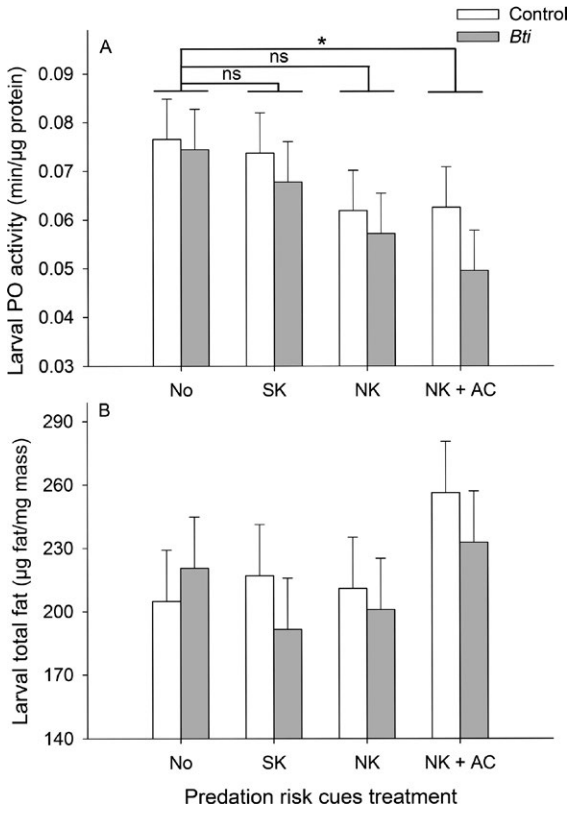

FIG. 4. (A) Phenoloxidase (PO) activity and (B) total fat of *Culex pipiens* larvae in response to *Bti* and four predation risk cues treatments: no chemical cues (No), synthetic kairomones of *Notonecta maculata* (SK), natural kairomones of *N. maculata* (NK), and natural kairomones of *N. maculata* + *C. pipiens* alarm cues (NK + AC). PO activity is expressed per minute and µg protein. Total fat is expressed in µg fat/mg wet mass. Jar means are given with 1 SE (based on 10 jar replicates). Lines represent contrast analyses explained in *Results*.

vector competence. A shorter adult life span of even a few days will reduce the likelihood of pathogens completing their incubation period (Thomas and Read 2007). For example, the incubation period for West Nile virus is ~5 d at 22°C in *Culex* mosquitoes (Kilpatrick et al. 2008), while the mean lifespan of adult *Culex* under summer field conditions in the absence of predation risk is only ~7 d (Lebl et al. 2013).

Our study is the first to evaluate and demonstrate the effects of synthetic kairomones on life history and physiology in the larvae (the actual prey) with carry-over effects to the adult stage. A study by Silberbush et al. (2010) had already proven that female mosquitoes avoided oviposition to a considerable extent in water with synthetic *Notonecta* kairomones. A key finding of our study was that responses to synthetic and natural kairomones were to a large extent similar. Indeed, the presence of synthetic and natural kairomones had similar carry-over effects to the adult stage by reducing PO activity and similar synergistic effects on mortality when combined with *Bti*. Given that we used the same concentration and ratio of the two identified kairomones present in *Notonecta* predators in the treatments with synthetic and natural kairomones, this suggests these two compounds are the major *Notonecta* kairomones. However, subtle differences between the effects of natural and synthetic kairomones existed and this translated in the synergistic effect at the level of the composite index of population performance only being present for the synthetic kairomones.

Responses to isolated exposure to Bti

Exposure to the sublethal *Bti* concentration had very little effect on life history and physiology in larval and adult mosquitoes when used in isolation. The only detectable *Bti* effect was a slight modification of development time with females slightly accelerating and males decelerating development. Any shortening of the development time may be an adaptive response to escape pesticides in the aquatic stage and has been observed in other aquatic insects (see e.g., Janssens and Stoks 2013b). Possibly males with their already much shorter development times had a lower need and were not able to further accelerate development under *Bti* exposure.

Responses to combined exposure to Bti and predation risk cues

The most striking finding of our study was that while neither *Bti* nor predation risk cues affected mortality, the combination of both stressors increased mortality by an average of 133% compared to the situation with only predation risk cues present, indicating a synergism (sensu Folt et al. 1999). Lethal synergistic interactions between predation risk cues and chemical pesticides have been observed before (e.g., carbaryl, Relyea and Mills 2001, Relyea 2003, fipronil, Qin et al. 2011). However, this interaction with predation risk cues has never been documented for biological pesticides. Moreover, this is the first demonstration that such interaction may occur with synthetic kairomones.

Also, when integrating the effects on the different life history traits into the composite index of population performance (Livdahl and Sugihara 1984), a synergistic interaction between *Bti* and predation risk cues was revealed. Population performance was only reduced in the presence of *Bti* when it was combined with synthetic kairomones or with the cocktail of natural kairomones and alarm cues. These results follow the synergistic pattern observed for mortality, except that no significant reduction in performance was detected when *Bti* was combined with natural kairomones. The loss of the synergism on r' for the treatment with natural kairomones is mainly caused by subtle, non-significant differences between the three predation risk cues treatments: wing length being somewhat lower for the treatment with natural kairomones in the absence of *Bti* and mortality being somewhat lower for the treatment with natural kairomones in the presence of *Bti*.

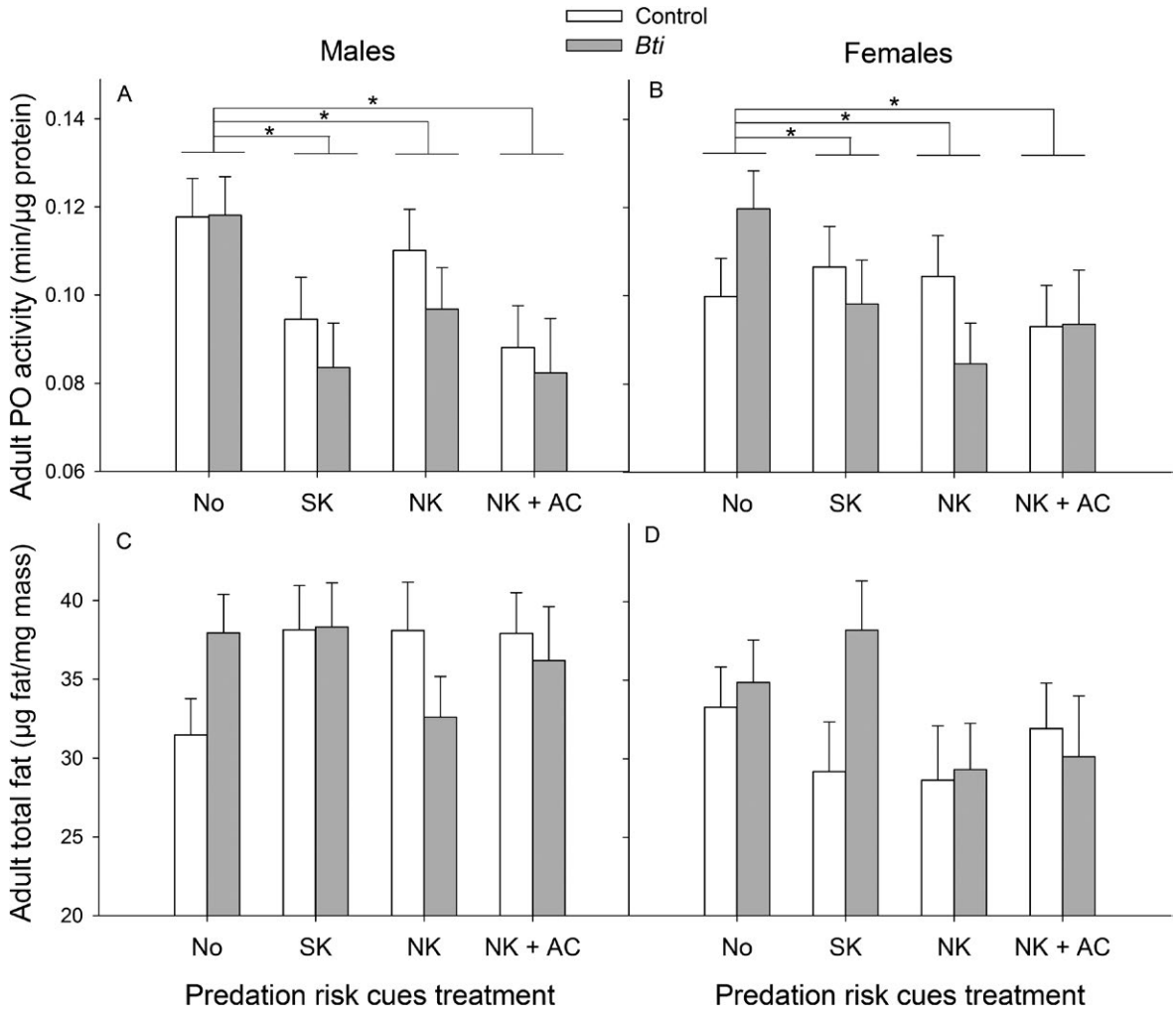

FIG. 5. (A, B) Phenoloxidase (PO) activity and (C, D) total fat of *Culex pipiens* mosquitoes in response to *Bti* and four predation risk cues treatments: no chemical cues (No), synthetic kairomones of *Notonecta maculata* (SK), natural kairomones of *N. maculata* (NK), and natural kairomones of *N. maculata* + *C. pipiens* alarm cues (NK + AC). PO activity is expressed per minute and µg protein. Total fat is expressed in µg fat/mg mass. Given are jar means with 1 SE for (A, C) males and (B, D) females. Lines represent contrast analyses explained in the results section.

A synergism between *Bti* and predator cues may seem surprising as *Bti* has a completely different mode of action compared to the chemical pesticides known to produce a lethal synergism. The endotoxins of *Bti* cause pore formation and cell lysis in the mosquito midgut, while carbamates (e.g., carbaryl) work by inhibiting acetylcholinesterase (Relyea 2003) and phenylpyrazoles (e.g., fipronil) work as GABA-gate chloride channel inhibitors (Qin et al. 2011). Yet, in a study specifically trying to relate the mode of action of chemical pesticides to the occurrence of a synergism with predator cues also no general pattern was present (Qin et al. 2011). A possible general mechanism not directly related to the mode of action of pesticides could be that the predation risk cues treatment causes a lower energy intake and/or a higher energy demand. When at the same time pesticides are present, the energy in the organism may not be sufficient for detoxification and repair (Relyea and Mills 2001, Campero et al. 2007, Qin et al. 2011).

Conclusions and applied perspectives

The control of vector mosquitoes to fight nuisance and diseases is one of the biggest challenges facing humankind (Garrett 2013, Alphey 2014) with the use of chemical pesticides often leading to environmental impact and the evolution of resistance (Heckel 2012, Köhler and Triebskorn 2013, Alphey 2014). While the biological pesticide *Bti* due to its high specificity may largely offset these problems, several recent studies did document negative effects on non-dipterans (e.g., Boisvert and Boisvert 2000, Poulin et al. 2010, Lajmanovich et al. 2015, but see Lagadic et al. 2014) and the evolution of resistance (e.g., Boyer et al. 2012). The here identified novel synergism type between a

biological pesticide and (synthetic) predator kairomones may open possibilities to counter these unwanted effects and thereby strengthen the use of *Bti* in IPM programs hereby creating more efficient and sustainable mosquito control. At the same time, this synergism highlights that good knowledge of the effects of biological pesticides and their interaction with other stressors may be necessary to translate the effects of biological pesticides under environmentally realistic conditions. It is, however, important that future research evaluates this synergism under more natural conditions.

The lethal synergistic interaction with the synthetic kairomones may allow lowering the recommended *Bti* concentrations for vector control; this can be especially interesting in small container habitats where *Culex* larvae are abundant and *Notonecta* predators are absent. Moreover, also the immunosuppressive effect and possible fitness effects (on longevity) of the exposure to the kairomone may affect vector competence, but further research is needed to be able to predict the net outcome of these effects. The application of lower *Bti* doses will help reaching two important applied goals. Firstly, it will lower potential harmful effects of *Bti* on non-target organisms and the resulting impact on the environment. For instance, non-biting midges (Chironomidae) which make up an important part of the biomass in shallow lakes and are important prey for fish in the larval stage, and for birds in the terrestrial stage have been identified as non-target organisms experiencing negative effects of *Bti* (e.g., Liber et al. 1998). Negative effects of *Bti* on those non-target organisms may thereby negatively affect bird populations (Poulin et al. 2010). Secondly, lower *Bti* doses may reduce the risk and delay the development of resistance against *Bti* (Heckel 2012). Even partial resistance to *Bti* is of increasing concern, as it will lead to the use of higher application doses and a switch to more harmful chemical pesticides. For the identified nonlethal effects on life history and physiology to affect vector competence in the field and eventually contribute to vector control, it will, however, be necessary to considerably reduce current application doses that are so high that lethal effects of *Bti* dominate and overrule any sublethal effects.

We have provided the important proof of principle under controlled laboratory conditions that even sublethal low *Bti* concentrations may impose a considerable mortality increase in *Culex* mosquitoes when combined with predator kairomones; and importantly, this synergism could be invoked by the synthetic kairomones of *Notonecta* predators. Moreover, the patterns in the composite index of population performance suggest that the combination of *Bti* with synthetic kairomones can negatively influence population growth rates of mosquitoes. Future research should focus on (1) the degree to which currently recommended *Bti* concentrations can be lowered while maintaining efficient vector control by combining them with these synthetic kairomones under natural field conditions and (2) on how cocktails of *Bti* and synthetic kairomones could be manufactured. For instance, new formulations of *Bti* enriched with synthetic kairomones could be developed. *Bti* is the only larvicide allowed in Europe for mosquito control (Directive 98/8/EC), and yearly many tons are applied, for example between 1981 and 2009 1000 tons of different *Bti* formulations were used on the Rhine flood plains in Germany (Becker et al. 2010). The EU demands the combination of different control strategies (IPM) and that all available techniques should be used in order to prevent the development of resistance to a certain pesticide (Directive 98/8/EC). The application of the here-documented novel synergism between *Bti* and synthetic kairomones would match these demands and may be a promising enrichment for current mosquito control although further research on the effects of these synthetic kairomones in more natural environments and their effects on non-target organisms is needed.

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